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Establishment of diagnostic test for enteric diarrhea in pigs using efficient multiplex polymerase chain reaction

SEUNGTAE KANG[1], NEELESH SHARMA[2], HEE SEOG KANG[3], GEE YOUNG MOON[4], SANG-KYOO OH[5], SANG-YI PARK[6], JU YEUN LEE[7] and DONG KEE JEONG[8]

Jeju National University, Jeju, 690 756 Republic of Korea

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ABSTRACT

Present study was planned to develop and validate a new methodology using multiplex PCR (M-PCR) assay for the detection of maximum number of diarrhea causing bacteria from pig faeces samples in a single test. Two M-PCR assays were designed to identify the diarrheic fecal bacteria and amplified the target genes including control group. Moreover, the unique primers were designed for the simultaneous detection of enterotoxigenic *E. coli* (ETEC; F4, F5, F6, F41, LT, STa, STb), *Salmonella* Enteritidis and *Salmonella* Typhimurium. The designed primers showed higher specificity and sensitivity to detect the targeted genes. The M-PCR method was able to detect at the 1 µg concentration of microbial DNA for all the genes detected by the designed primers. Further, the efficacy of M-PCR was investigated using 3 non-*E. coli* pathogenic strains *L. intracellularis, B. hyodysenteriae*, and *Salmonella* from different pig farms. Results sufficiently revealed that the developed M-PCR assay is an efficient tool for detection of ETEC and other non-*E. coli* strains from naturally infected pigs. The ability of M-PCR to identify the many bacteria simultaneously without biochemical identification would facilitate early diagnosis and treatment of diarrhea in pigs.

Key words: Collibacillosis, Multiplex-PCR, Pig diarrhea, Salmonellosis

Gastrointestinal diseases, among the major cause of death for suckling piglets (Sanz *et al.* 2001), are responsible for 31% and 37% of the total mortality during the neonatal and post-weaning periods (de la Fe Rodriguez *et al.* 2013). Several distinct pathogenic bacterial species may be responsible for bacterial diarrhea in pigs, of which the few are, most common and/or economically important such as enterotoxigenic *Escherichia coli* (ETEC), the agent of colibacillosis; *Salmonella* species, the agent of porcine salmonellosis (PS); *Lawsonia intracellularis*, the agent of proliferative enteropathy (PE); and *Brachyspira hyodysenteriae*, the agent of swine dysentery (SD) (Collins *et al.* 2000, Lee *et al.* 2008).

ETEC strains caused the diarrhea due to 2 kinds of virulence factors, fimbrial adhesions and enterotoxins (Nagy and Fekete 1999). There are various types of fimbriae and enterotoxins associated with ETEC in different host species. The common fimbriae associated with ETEC in swine are F4 (K88), F5 (K99), F6 (987P), and F41 (Nagy and Fekete 1999, DebRoy and Maddox 2011). The common enterotoxins are heat-stable (STa, STb) and heat-labile (LT) (Nagy and Fekete 1999). Fimbriae attach to microvilli of intestinal epithelial cells, colonize the mucosal surface of the small and produce enterotoxins, resulting diarrhea.

The developments of PCR-based diagnostic techniques, including multiplex PCR (M-PCR), have greatly improved the sensitivity, specificity and speed of detecting microorganisms (Mothershed and Whitney 2006). M-PCR is able to amplify multiplex targets including virulence factors (Severgnini *et al.* 2011). The M-PCR technique does not require expensive machines and is a rapid, economical, highly sensitive and specific method for the detection of the pathogenic bacteria. It is an efficient diagnostic tool, which is valuable for epidemiological surveillance and confirmed diagnosis of multiple bacterial infections including toxins in swine herds. The currently developed M-PCR method will be useful for a faster diagnosis and study of bacterial diarrhea in pig herds. This study aimed to develop and validate a methodology for the simultaneous detection of enteropathogenic *E. coli* (EPEC; F4, F5, F6, F41, LT, STa, STb), *Salmonella* Enteritidis and *Salmonella* Typhimurium to reduce the number of tests needed for

Present address: [1,2,8] Department of Animal Biotechnology (kost1212@nate.com, drneelesh_sharma@yahoo.co.in, newdkjeong@gmail.com), Faculty of Biotechnology, [3,4]Informind (keystone@infomind.co.kr, jymun14@infomind.co.kr), Jedong Happy Zone Bldg, 92, Seosa-ro, Jeju-si, Jeju-do, 690–812, Republic of Korea. [5]Jinwoo Soft Innovation (mrosang @jinwoosi.co.kr), Jeju Venture Maru, Jungang-ro 217, Jeju-City, Jeju-Do 690–827, Republic of Korea. [6]TLC Technology Co. Ltd. (sypark @tlctech.co.kr) 38, Sagimak 1-gil, Gwacheoun-Si-Gyeonggi-Do, Republic of Korea. [7]TNT research (foggygirl @naver.com), Co. Ltd., 924 Doosan Venture Digm, Pyeongchon-dong, Dongan-gu, Anyang-si, Gyeonggi-do, Republic of Korea.

diagnosis of diarrheagenic bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions: A panel of reference bacterial strains- *Salmonella* Enteritidis (SE), the *Salmonella* Typhimurium (ST), 7 kinds of enteropathogenic *E. coli* - the Colibacillosis F4 (K88), the Colibacillosis K99 (F5), the Colibacillosis P987 (F6) and the Colibacillosis F41, the heat labile (LT), and heat stable toxin STa and STb, were procured from the National Institute of Animal Pathogen, Korea, used for the multiplex PCR system. All strains of *Salmonella* and *E. coli* were grown aerobically in Luria-Bertani (LB) broth and incubated at 37°C with shaking at 220 rpm for 18–20 h.

Bacterial DNA extraction: The DNA was extracted from the procured bacterial strains after amplifying the LB broth. The DNA was isolated from colonies by suspending the colonies in 50µl of deionized water and centrifuged separately at 15,000 rpm for 20 min. After getting the pellet followed the plasmid extraction kit protocol, and finally dissolved the extracted bacterial DNA in deionized water. DNA quality and concentration was measured using the spectrophotometer at 260 nm, and stored at –20°C. Extracted and purified DNA was used as template for the PCR and M-PCR.

Primer design: The newly synthesized and previous study primers for specific amplification of *Salmonella* Enteritidis (SE), *Salmonella* Typhimurium (ST), 7 kinds of Enteropathogenic *E. coli* - the Colibacillosis K88 (F4), the Colibacillosis K99 (F5), the Colibacillosis P987 (F6) and the Colibacillosis F41, the heat labile (LT), and heat stable toxin STa and STb are described in Table 1. A separate set of primers was also synthesized for diarrheic feces bacteria- *Lawsonia intracellularis, Brachyspira hyodysenteriae* and *Salmonella* species (Table 2). The primers were commercially synthesized and tested for PCR and M-PCR with DNA extracted from bacterial strains alone or in combinations. The specificity of each primer was confirmed by monoplex PCR.

Polymerase chain reaction (PCR): PCR was performed in eppendorf mastercycler gradient using specific primers of targeted bacteria. The PCR reaction mixture consisted total 20 µl volume using 1 µl DNA template, 2.5U Taq polymerase, forward and reverse primers (10 pmol/µl each), 2 µl 10x reaction buffer (100mM Tris-HCl), 2 µl 25 mM $MgCl_2$, 0.5 µl 10 mM deoxyribonucleotides (dATP, dGTP, dCTP, dTTP) and following temperature-time programme: 95°C for 10 min, 94°C for 40 sec, 60°C for 40 sec, 72°C for 45 sec for 30 cycles and 72°C for 10 min in the last cycle. The amplified products were separated by

Table 1. Primer sequences and predicted size of amplification products

Organism	Oilgonucleotide sequences of primers (5′–3′)	Product size (bp)	Reference
Colibacillus F4(K88)	GCTGCATCTGCTGCATCTGGTATGGCCACTGAGTGCTGGTA GTTACAGCC	792	31
Colibacillus F5(k99)	TGCGACTACCAATGCTTCTGTATCCACCATTAGACGGAGC	450	23
Colibacillus F6(P987)	TCTGCTCTTAAAGCTACTGGAACTCCACCGTTTGTATCAG	333	23
Colibacillus F41	GAGGGACTTTCATCTTTTAGAGTCCATTCCATTTATAGGC	431	23
Colibacillus LT	ATTTACGGCGTTACTATCCTCTTTTGGTCTCGGTCAGATATG	280	24
Colibacillus STa	TCCGTGAAACAACATGACGGATAACATCCAGCACAGGCAG	244	23
Colibacillus STb	GCCTATGCATCTACACAATCTGAGAAATGGACAATGTCCG	279	23
Salmonella Enteritidis	CATTGGTGATGGTCTTGTCGCTCGCCTTTGCTGGTTTTAG	298	This Study
Salmonella typhimurium	AGCCGCTCAGTATTGAGGAAGGACACGACTTCATCGGAAT	323	This Study
Colibacillus F4(K88)	CTGGTGATTTCAATGGTTCGGACCGTTTGCAATACCCAGT	702	This Study
Colibacillus F5 (K99)	TTGGGCAGGCTGCTATTAGTTCATAGATATGCCCGCAATG	422	This Study
Colibacillus F6 (987P)	CTGCCAGTCTATGCCAAGTGTACGGTGTACCTGCTGAACG	460	This Study
Colibacillus F41	AGCAGCGAAGATGAGTGATGTGCCCTGGCATTAACTTTTC	393	This Study
Colibacillus LT	TTATGATCACGCGAGAGGAATGCTCAGATTCTGGGTCTCC	500	This Study
Colibacillus STa	TTGTAATCCTGCCTGTGCTGACCAGACGGTTCAGATGAGG	152	This Study
Colibacillus STb	TTGCAGCAAAAGGATGCTAATGGACGGGAATCTTCAAAAG	197	This Study

Table 2. Primer sequences and predicted size of amplification products

Organism	Oilgonucleotide sequences of primers (5‘–3‘)	Targeted gene	Product size (bp)	Reference
L. intracellularis	GCAGCACTTGCAAACAATAAACTTTCTCCTTTCTCATGT CCCATAA	rRNA	210	29
B. hyodysenteriae	GCTGGAGATGATGCTTCTGGGTCCAAGAGCTTGGCTGTTC	NADH oxidase	403	29
Salmonella spp.	TTGGTGTTTATGGGGTCGTTGGGCATACCATCCAGAGAAA	*invA*	298	29
L. intracellularis	TGTCAAACACTCCGTGCTGTTACTTGTCCCTGCACCTCCT	rRNA	241	This Study
B. hyodysenteriae	CGTGGTTTACGTATGGCTGAGTCCAAGAGCTTGGCTGTTC	NADH oxidase	367	This Study
Salmonella spp.	CATTGGTGATGGTCTTGTCGCTCGCCTTTGCTGGTTTTAG	*invA*	298	This Study

electrophoresis in 2% agarose gel and stained with ethidium bromide.

Multiplex PCR (M-PCR) reaction: PCR was performed in eppendorf mastercycler gradient using specific primers of targeted bacteria. The M-PCR reaction mixture consisted total 20 µl volume using 1 µl DNA template, 2.5U Taq polymerase, 0.5 µl forward and reverse primers (10 pmol/µl each), 2 µl 10x reaction buffer (100mM Tris-HCl), 2 µl 25 mM $MgCl_2$, 0.5 µl 10 mM deoxyribonucleotides (dATP, dGTP, dCTP, dTTP) and following temperature-time programme: 95°C for 10 min, 94°C for 40 sec, 60°C for 40 sec, 72°C for 45 sec for 35 cycles and 72°C for 20 min in the last cycle. The amplified products were separated by electrophoresis in 2% agarose gel and stained with DNA markers.

Analysis of sensitivity for the multiplex PCR: To evaluate the effect of the number of bacterial cells on the sensitivity and specificity of the M-PCR method, purified DNA from 9 bacterial strains in various concentrations to optimize the final concentration of DNA template. The diluted multiplex PCR was performed according to a concentration including 100 pg, 1 ng, 10 ng, 100 ng, 1µg 2 µg and a band was checked to measure sensitivity of multiplex PCR.

Collection of the fecal sample and isolation of the DNA: Fecal samples were collected randomly in sterile bottles from pigs showing diarrheic symptoms from 4 pig farms, Jeju, Korea. DNA was extracted from feces using the DNA stool mini kit (La *et al.* 2003) and followed kit protocol. 0.1g piglets diarrhea feces inoculated in the LB broth and allowed to shake at 220 rpm for 18–20 h at 37°C. Bacterial culture was centrifuged at 15,000 rpm for 20 min. DNA quality and concentration was measured using the spectrophotometer at 260 nm, and stored at –20°C.

RESULTS AND DISCUSSION

Multiplex PCR for reference strains for detection: All the 7 *E. coli* strain, 2 strains from *Salmonellosis* species (*L. intracellularis*, and *B. hyodysenteriae*) gave specific amplification of the correct predicted product size. Diarrheagenic *E. coli* reference strains K88 (F4), K99 (F5),

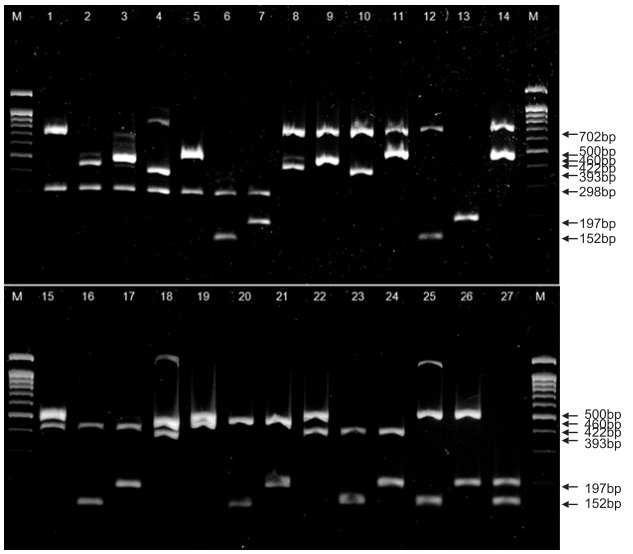

Fig. 2. Multiplex PCR for the individual or combination from the bacterial agents. Lane M, 100–bp molecular size ladder; lane 1, ST&F4; lane 2, ST&F5; lane 3, ST&F6; lane 4, ST&F41; lane 5, ST< lane 6, ST&STa; lane 7, ST &STb; lane 8, F4&F5; lane 9, F4&F6; lane 10, F4&F41; lane 11, F4< lane 12, F4&STa; lane 13, F4&STb; lane 14, F5&F6; lane 15, F5< lane 16, F5&STa; lane 17, F5&STb; lane 18, F6&F41; lane 19, F6< lane 20, F6&STa; lane 21, F6&STb; lane 22, F41< lane 23, F41&STa; lane 24, F41&STb; lane 25, LT&STa; lane 26, LT&STa; lane 27, STa&STb.

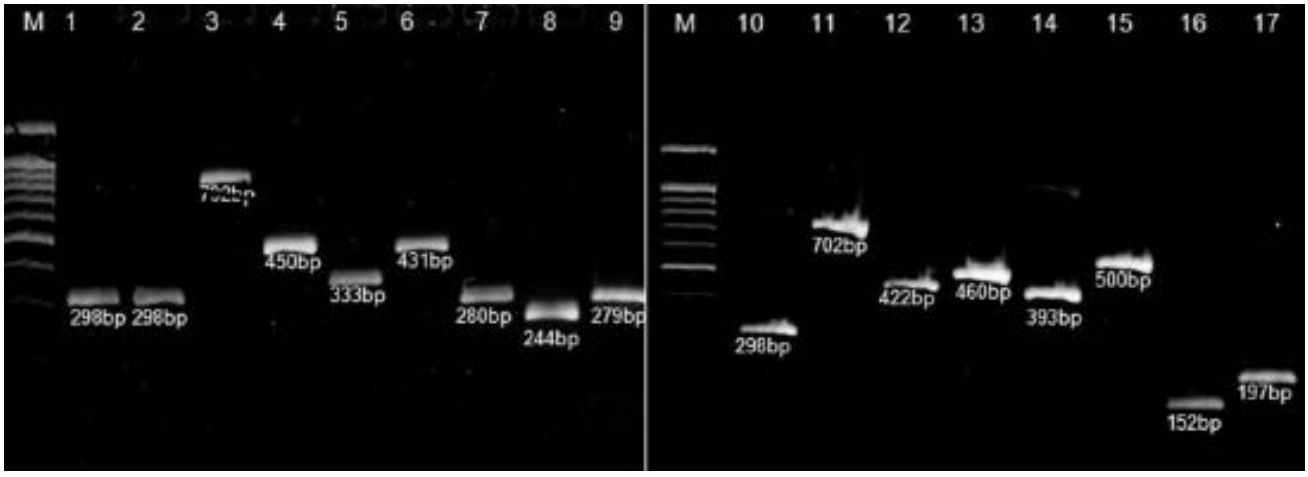

Fig. 1. Agarose gel electrophoresis of PCR products from *Salmonella* and *Escherichia coli*. Lane M, 100bp DNA ladder; lane 1, *Salmonella* Enteritidis; lane 2, *Salmonella* Typhimurium; lane 3, *Escherichia coli_F4*; lane 4, *Escherichia coli_F5*; lane 5; *Escherichia coli_F6*; lane 6, *Escherichia coli_F41;* lane 7, *Escherichia coli_LT;* lane 8, *Escherichia coli_STa*; lane 9, *Escherichia coli_STb*; lane 10, *Salmonella typhimurium*; lane 11, *Escherichia coli_F4*; lane 12, *Escherichia coli_F5*; lane 13, *Escherichia coli_F6*; lane 14, *Escherichia coli_F41;* lane 15, *Escherichia coli_LT;* lane 16, *Escherichia coli_STa*; lane 17, *Escherichia coli_STb*.

P987 (F6), F41 and H10407 (STa, STb and LT) were used as positive controls. This study also used *Salmonella* strains (*S.* Enteritidis and *S.* Typhimurium) to define the accuracy of the method. The amplification of the individual genes is shown in Fig. 1.

To detect species simultaneously, a mixture of 2 sets consisted of 9 primer pairs were used in 2 different multiplex PCR assays. The multiplex PCR assays showed specificity in identifying the reference strains (Fig. 2). Each of the 2 pairs of oligonucleotide primers exclusively amplified the target genes of the specific microorganisms. Further, the amplification of the DNA along and in the combination yielded products corresponded to the expected molecular weight of DNA from each species were confirmed by multiplex PCR (Fig. 3). The primers designed in the present investigation showed more efficacies then the pre-existing primer for the detection of the specific target genes.

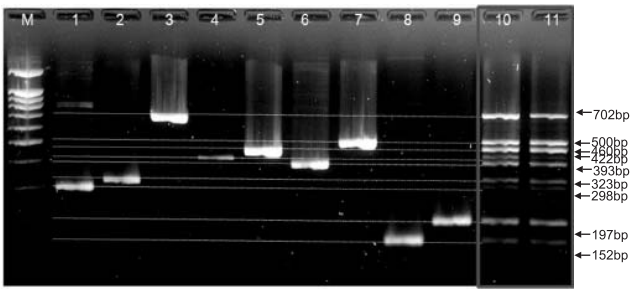

Fig. 3. Agarose gel electrophoresis of multiplex PCR amplification of nine target gene segments of *Salmonella* and *Escherichia coli*. Lane M, 100bp DNA ladder; lane 1, *Salmonella* Enteritidis; lane 2, *Salmonella* Typhimurium; lane 3, *E.coli_F4*; lane 4, *E.coli_F5*; lane 5, *E.coli_F6*. lane 6, *E.coli_F41;* lane 7, *E.coli_LT;* lane 8, *E.coli_STa*; lane 9, *E.coli_STb*; lane 10, Multiplex PCR, lane 11, Multiplex PCR.

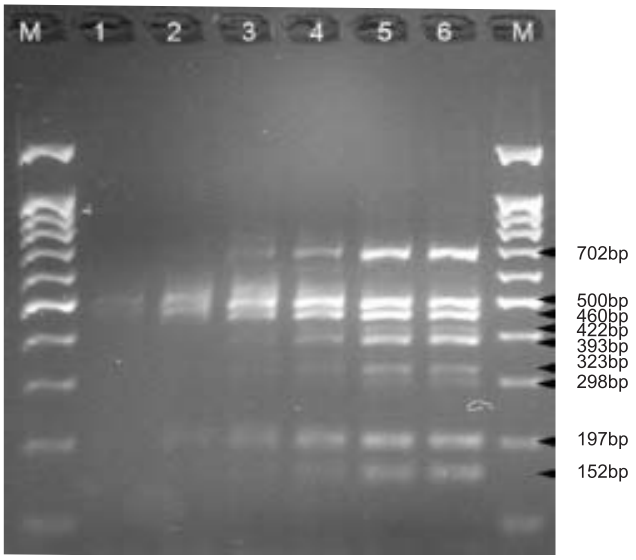

Fig. 4. Sensitivity of the multiplex PCR against nine target bacterial. Lane M, 50bp DNA step ladder; lane 1, 100 pg DNA; lane 2, 1 ng DNA; lane 3, 10 ng DNA; lane 4, 100ng DNA; lane 5, 1 µg DNA; lane 6, 2 µg DNA.

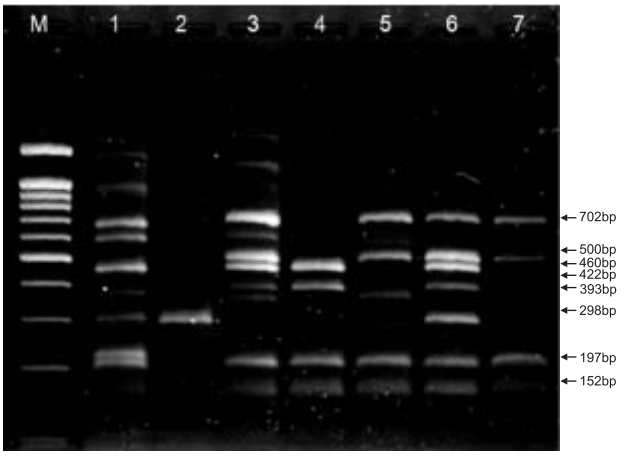

Fig. 5. Agarose gel electrophoresis of multiplex PCR amplification from the collected feces samples from field pigs. Lane M, 100bp DNA ladder; lane 1, sample 1; lane 2, sample 2; lane 3, sample 4; lane 4, sample 5; lane 5, sample 6, lane 6, sample 7*;* lane 7, sample 8.

Moreover, it did not show any nonspecific amplification products.

The sensitivity of the multiplex-PCR method was tested on different preparations. The multiplex-PCR method was able to detect at the 1µg concentration for all the genes detected by the primers, constructed in the present study (Fig. 4).

Analysis of field samples for diarrhea causing bacteria: To assess the reproducibility of current protocol, extended the study to analyze the clinical diarrheal pathogens from the 7 samples of pig faeces. To demonstrate the utility of the multiplex PCR assay, the purified DNA obtained from pure cultures of each bacterial agent alone or mixed in different combinations and concentrations from faeces specimens were amplified. Variations in the number of the organisms were found in the samples. The identification of the bacteria was carried out using our newly designed primer (Fig. 5). The prevalence of *E. coli* was found in maximum cases.

M-PCR for the detection of non-E. coli strains: The present investigation was also carried out to identify the non-*E.coli* strains using M-PCR. For each of the target genes, 2 sets of primers were used. The DNA fragments of approximately 241, 367, and 298 bp from the faeces samples and based on sequences in GenBank database, the sizes of these amplification products were consistent with the predicted sizes for rRNA of *L. intracellularis*, NADH oxidase gene of *B. hyodysenteriae* and *invA* gene of *Salmonella* spp. respectively (Fig. 6). Further the identity of those fragment were confirmed through DNA sequencing (data are not given).

To develop the multiplex PCR, present study tested the progressive incorporation of primers corresponding to the literature and several combinations of melting temperatures and primer concentrations. Nonspecific amplification products were not detected by M-PCR assay of purified

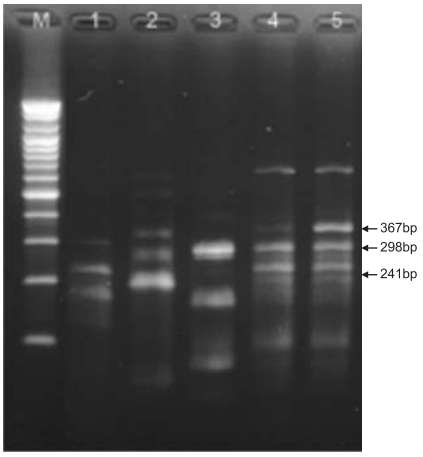

Fig. 6. Agarose gel electrophoresis of multiplex PCR amplification of *Lawsonia intracellulraris, Serpulina hyodysenteriae, Salmonella* Enteritidis. Lane M, 100bp DNA ladder; lane 1, *Lawsonia intracellulraris* ; *Serpulina hyodysenteriae*; lane 3, *Salmonella* Enteritidis; lane 4, multiplex PCR; lane 5, multiplex PCR.

DNA from *L. intracellularis, B. hyodysenteriae*, and *Salmonella* sp. The efficacy of the newly designed primers showed more sensitivity to detect the target genes.

Diarrhea caused by enteric infections is a major factor of morbidity and mortality in pigs worldwide (Farzan *et al.* 2013). Colibacillosis is the major cause of illness and the death in the neonatal and recently weaned pigs (Francis 1999). The prevalence and other epidemiological features of diarrhea causing pathogens are varying from region to region. ETEC has widespread distribution and widely use of antibiotics in piggeries in Korea (Lee *et al.* 2009). The documentation on the identification of enterotoxigenic *E. coli* from pigs between 2002 and 2007, 188 *E. coli* strains were isolated from piglets of suckling and weaning periods with diarrhea on different farms in South Korea (Lee *et al.* 2008).

An increasing number of investigators have employed the tools of molecular biology to facilitate the diarrhea diagnostic process. Numerous PCR methods have been developed to identify the virulence genes (Kaper *et al.* 2004). To avoid the difficulty of performing phenotypic assays, the multiplex PCR is a practical, promissive and rapid diagnostic tool for identification of diarrheic bacteria in a single reaction tube (Toma *et al.* 2003). Sensitivity and specificity assays were investigated by testing different strains. Current protocol showed high sensitivity and specificity to detect the diarrhea causing bacteria from feces samples from pig using control strains by the multiplex PCR assays. *E. coli* reference strains K88 (F4), K99 (F5), P987 (F6), F41 and STa, STb and LT were selected as positive controls. The maximum numbers of enterotoxigenic *E. coli* were identified from the collected samples. The research suggested that enterotoxigenic *E. coli* (ETEC) is the main cause of diarrhea to pigs including heat labile exterotoxin (LT), and heat stable enterotoxins STa and STb (Francis 1999, Osek 2001). The fimbrial adhesins are produced include K88, K99, 987P, F41, and F18, that mediate the adherence of the bacterium to the mucosal surface (Lee *et al.* 2009, Chen *et al.* 2014).

Further, efficacy of M-PCR on detection of non- *E. coli* stains was investigated. We designed specific primers for the rRNA of *L. intracellularis*, NADH oxidase gene of *B. hyodysenteriae* and *invA* gene of *Salmonella* sp. Amplification of the targeted genes from the 3 different pathogens was confirmed by previously reports with the individual PCR assay (Atyeo *et al.* 1999, Stone *et al.* 1994). Under the described conditions nonspecific amplifications product could not be detected in the M-PCR test. The levels of detection in our study are consistent with or slightly better than those previously reported for the individual species-specific PCRs (Baccaro *et al.* 2003, La *et al.* 2003).

In the present study, the unique combination of multiple primer sets of 3 important enterotoxin variants responsible for diarrhea and simultaneously differentiation of *E. coli* from non-*E. coli*, greatly improved the detection efficiency and specificity and eliminated the need for subsequent confirmation procedures. Our results demonstrated the high discriminatory application of the multiplex PCR assay and the potential of the test for rapid and specific identification and toxin profiling of *E. coli* strains responsible for diarrhea.

In conclusion, the multiplex PCR system described here is an efficient tool for detection of ETEC other non-*E coli* strains from naturally infected pigs feces. The M-PCR could prove useful for the rapid, sensitive and specific detection of these 3 important enteric pathogens in porcine fecal samples. The ability to analyze and identify the bacterial isolates without biochemical identification should facilitate diagnosis and treatment of diarrheal disease due to ETEC and other pathogenic groups. The field application would be beneficial to reduce transmission of diarrheagenic infection on swine farms and help in the selective and cost effective treatment.

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